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# Functional Expression of Rat Renal Na/$P_i$-Cotransport (NaPi-2) in Sf9 Cells by the Baculovirus System

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**Abstract.** The recently cloned Na/$P_i$-cotransport system NaPi-2 is an apical membrane protein of rat proximal tubular cells and is involved in proximal phosphate reabsorption. To make the protein available for further functional/structural studies, this transport system has been expressed in Sf9 insect cells using a recombinant baculovirus. Sf9 cells infected with NaPi-2 (or 6His tagged NaPi-2) expressed functional Na/$P_i$-cotransport up to 20- to 50-fold over noninfected Sf9 cells. Transport of phosphate in infected cells was highly dependent on sodium, exhibited a $K_m$ for $P_i$ of 0.114 mM and an apparent $K_m$ for Na of 63 mM (Hill coefficient of approximately 3) and was stimulated by high external pH. Infected cells expressed a polypeptide of 65 kDa representing a nonglycosylated form of the 85 kDa mature NaPi-2 transporter as present in proximal tubular brush-border membranes. By confocal microscopy expression of NaPi-2 protein was observed in the plasma membrane, yet submembranous accumulation of NaPi-2 protein could not be excluded. This demonstrates that the rat proximal tubular Na/$P_i$-cotransport system NaPi-2 can be successfully expressed in Sf9 cells with characteristics similar to that in isolated brush-border membranes. The 6His tagging will permit isolation of the NaPi-2 cotransporter in amounts sufficient for structural/functional studies.

**Key words:** Renal Na/$P_i$-cotransport — Sf9 cells — Infection

## Introduction

By expression cloning, starting from a rat kidney cortex cDNA-library, we have cloned and described a sodium-dependent transport system for phosphate (Na/$P_i$-cotransporter, NaPi-2; 16). Functional characterization of the NaPi-2 cotransporter by expression in oocytes of Xenopus laevis [16] and studies on the nephron localization of NaPi-2 mRNA and of the NaPi-2 protein [7] suggested that the cotransport system NaPi-2 is related to proximal tubular apical Na/$P_i$-cotransport. Furthermore, evidence was obtained that the NaPi-2 related transport system is involved in the physiological regulation of proximal tubular $P_i$-reabsorption, such as by a low $P_i$-diet [22].

The baculovirus Sf9 cell expression system is suitable for the functional expression of a variety of heterologous proteins [8, 9, 12; for review *see* ref. 15]. An oocyte independent expression system permitting functional characterization would first provide further evidence for the NaPi-2 cotransporter being a proximal tubular Na/$P_i$-cotransporter and secondly would eventually allow to obtain quantities of pure NaPi-2 protein required for further biochemical analysis, e.g., membrane topology.

Here we show that the NaPi-2 protein, wild type or tagged at the N-terminus with 6 histidine residues, when expressed in Sf9 cells by the use of a recombinant baculovirus is functionally active and that expressed Na/$P_i$-cotransport activity exhibits similar transport characteristics as described for proximal tubular apical Na/$P_i$-cotransport. Therefore the Sf9-baculovirus expression system may be a source of high amounts of functionally active renal Na/$P_i$-cotransporters for future studies on structure/function relationships of this membrane protein.

## Materials and Methods

### Plasmid Construction

From the plasmid pSport1 containing NaPi-2 cDNA [16] the entire coding region was isolated by digestion with NotI and EcoRI. The

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isolated insert was ligated into the baculovirus expression vector pVL 1393 (Pharmingen, San Diego, CA) such that the expression of the NaPi-2 protein in Sf9 cells will occur under the control of the polyhedrin promotor [18]. Similarly, the isolated NaPi-2 insert was ligated into the baculovirus expression vector pAcSG-His NT-B (Pharmingen) to obtain an N-terminal 6His tagged NaPi-2 protein. Clones containing the NaPi-2 inserts in the proper orientation relative to the polyhedrin promotor were identified by dideoxy sequencing and plasmids were purified by CsCl-gradient centrifugation.

### Production of Recombinant Virus

The insect cell line Sf9 (derived from *Spodoptera frugiperda*) was maintained in supplemented Grace's Medium (Gibco, BRL) containing 10% fetal bovine serum (Flow Laboratories) and 50 μg/ml Gentamycin (Gibco, BRL) in 75 $cm^2$ tissue culture flasks at 27°C.

To generate recombinant baculoviruses, Sf9 cells ($2 \times 10^6$ cells per 60 mm petri dish) were transfected according to the manufacturers instructions with 2 μg of the NaPi-2 constructs (wild type or 6 His tagged) and 0.5 μg of a modified Baculovirus (*Autographica californica*) viral DNA (Baculogold, Pharmingen, San Diego, CA) which contains a lethal deletion that is replaced by the DNA of the transplacement plasmid. After 2–3 days, cell culture supernatants were collected and screened for recombinant baculoviruses by hybridization (Southern dot blots) using a full-length NaPi-2 cDNA probe. To clone recombinant baculoviruses, Sf9 cells were infected with serial dilutions of viruses and after 2–3 days again tested by Southern blotting.

For routine infection, approximately $5 \times 10^5$ cells/35 mm dish (grown to log phase) were infected with recombinant viruses at a MOI of 0.1 to 1. After 1 hr of incubation, the medium (0.5 ml) was replaced with 2 ml of fresh virus-free medium. For most experiments described, cells were harvested 4–5 days after infection.

### SDS-polyacrylamide Gelelectrophoresis and Western Blotting

Sf9 cells were infected with recombinant baculoviruses (*see above*) and at various time points of postinfection cells were washed once with PBS and solubilized with RIPA (mM) 10 Tris/HCl (pH 8.0), 1 EDTA, 150 NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS) and the protease inhibitors *p*-methylsulfonylfluorid (0.7 mM) and leupeptin (20 μg/ml). Cell lysates were denaturated in 4% SDS. Proteins were separated by SDS-polyacrylamide gelelectrophoresis (9% gels; 13) and transferred onto nitrocellulose (Schleicher & Schuell, BA 83) according to [21]. Blots were blocked with 150 mM NaCl, 20 mM Tris/HCl (TBS; pH 7.3) containing 5% w/v low fat milk powder and 1% Triton X-100 (Blotto) for 2 hr and then incubated overnight with rabbit anti(NaPi-2)antisera at a dilution of 1:4000. The antisera used were raised against synthetic *N*-terminal and C-terminal NaPi-2 peptides and have been characterized recently [7]. Afterwards, blots were washed three times with 1:10 diluted blotto and incubated for up to one hr with fresh blotto solution. To visualize primary antibody binding, goat anti-rabbit IgG alkaline phosphatase conjugates (BioRad) were added for additional 2 hr. Finally, blots were washed three times with TBS and color reaction was performed with the chromogens nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BioRad, Hercules, CA).

### Confocal Microscopy

Sf9 ($1.2 \times 10^5$ cells) were placed onto sterile 22 × 22 mm glass coverslips in 35 mm culture dishes and allowed to attach overnight. The supernatant was removed and replaced by 500 μl of medium containing recombinant viruses at a MOI of 0.1. After 1 hr of incubation, the medium was replaced with 2 ml fresh virus-free medium and cells were incubated for 4 days. Then cells were fixed for 20 min with 3% paraformaldehyde in PBS. After two washes with PBS, excess paraformaldehyde was quenched by the addition of PBS containing 20 mM glycine for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After a further wash, cells were incubated for 1 hr with 2% low fat milk powder, 0.05% Triton X-100 in PBS and subsequently incubated with the primary antibody at a dilution of 1:300 for 2 hr. After three washes with PBS containing 0.05% Triton X-100, FITC-labeled secondary antibodies (swine anti-rabbit IgG, Dakopatts, Denmark) were added in blocking buffer at a dilution of 1:75 and incubated for 1 hr at room temperature in the dark. Finally, cells were washed as above and the coverslips were embedded with glycergel (Dakopatts). Images were analyzed on a Zeiss Laser Scan microscope under a 63× objective. Images were averaged over at least eight scans.

### Phosphate Uptake Measurements

Infected and noninfected cells grown as described above were analyzed for Na-dependent transport of phosphate at day 4 of postinfection. Cells were washed once with substrate-free uptake medium (*see* ref. 3) and incubated (if not otherwise stated, *see* figure legends) with 1 ml of uptake medium containing 0.1 mM $^{32}P$-$K_2HPO_4$ (1 μCi/ml; NEN 053). After various time points of incubation at 25°C, the uptake medium was sucked off and cells were washed four times with ice-cold stop solution (*see* ref. 3). After solubilization with 0.5 ml of 1% Triton X-100, aliquots were used for the determination of radioactivity by liquid scintillation counting and for determination of the protein content according to a modification of the Lowry method [14]. To determine Na-independent transport of $P_i$, sodium was replaced equimolarly by *N*-methyl-glucamine.

For the determination of the apparent Km for $P_i$, extracellular concentration was varied between 0.05 and 1 mM; the pH value was kept constant at 7.4. For the determination of the interaction constant with sodium ions, NaCl was equimolarly replaced with *N*-methyl-glucamine/HCl. The Tris/Hepes buffer system was used throughout the pH-range used, between 6.5 and 8.0.

### Abbreviations

| | |
|---|---|
| $P_i$, | inorganic phosphate |
| cDNA, | complementary DNA |
| SDS, | sodium dodecyl sulfate |
| PBS, | phosphate-buffered saline |
| Tris, | tris(hydroxymethyl)aminomethane |
| EDTA, | ethylenediaminetetraacetic acid |
| TBS, | tris-buffered saline |
| MOI, | multiplicity of infection |
| FITC, | fluorescein isothiocyanate |
| BBMV, | brush-border membrane vesicle |
| PAGE, | polyacrylamide gel electrophoresis |
| cRNA, | complementary RNA |
| OK-cells, | opossum kidney cells |

## RESULTS

### Preparation of Recombinant Baculoviruses

The rat renal Na/$P_i$-cotransport system NaPi-2 was introduced into the genome of the baculovirus by the trans-

fer vectors pVL1393 and pAcSG-His NT-B in order to generate wild-type or N-terminally 6His-tagged NaPi-2 protein respectively. The final constructs contained the NaPi-2 cDNA, which included the full length coding region flanked by 53 bp at the 5′-untranslated region and 497 bp at the 3′-untranslated region [16]. The 6His-tagged NaPi-2 protein contained 27 additional amino acids between a thrombin cleavage site and the first methionine of wild-type NaPi-2 protein. Permissive Sf9 cells were cotransfected with above NaPi-2 plasmids and viral DNA containing a lethal mutation when not recombinated with the plasmid. Recombinant baculoviruses were detected by Southern blotting using an NaPi-2 cDNA probe (*data not shown*) and recombinant viruses were cloned according to described methods [18].

### Detection of Expressed NaPi-2 Protein

Expression of recombinant NaPi-2 protein was monitored by the use of two polyclonal anti(NaPi-2) antisera raised against synthetic C-terminal and N-terminal peptides of the deduced NaPi-2 amino acid sequence; both antisera have been characterized recently [7]. As shown in Fig. 1*A*, Sf9 cells infected with either the wild type or the 6His-tagged NaPi-2 cDNA expressed a protein of approximately 65 kDa detected by both antisera. No reaction using these antisera was observed in lysates of uninfected cells (Fig. 1) or in cells infected with wild-type baculovirus (*data not shown*). As recently demonstrated by Western blot analysis using isolated rat proximal tubular brush-border membranes, the mature NaPi-2 *N*-glycosylated protein [7, 10] migrates as an 80 to 90 kDa protein (*see* Fig. 1*A*). The difference observed in the mobility of the NaPi-2 protein expressed in rat renal BBMVs compared to the NaPi-2 protein expressed in Sf9 cells suggests that in Sf9 cells, the NaPi-2 protein is poorly or not glycosylated since after an in vitro translation of NaPi-2 cRNA in the absence of pancreatic microsomes the unglycosylated form of the NaPi-2 protein migrates as a 70 kDa band [16]. Furthermore, after a treatment of BBMVs with endoglycosidase F the NaPi-2 protein migrated as a 70 kDa protein [10] again suggesting that in Sf9 cells the NaPi-2 protein is not (or poorly) glycoslated. To determine the time course of the NaPi-2 protein expression, whole cell-lysates were immunoblotted at various days after infection. As shown in Fig. 1*B*, expression of NaPi-2 protein was not detectable until day three but increased rapidly afterwards. Maximal expression was routinely observed after 4 to 5 days postinfection. Afterwards, the amount of the NaPi-2 protein in whole cell lysates varied considerably, most likely because of cell lysis.

Cellular localization of expressed wild-type NaPi-2 protein was analyzed by immunofluorescence (Fig. 2).

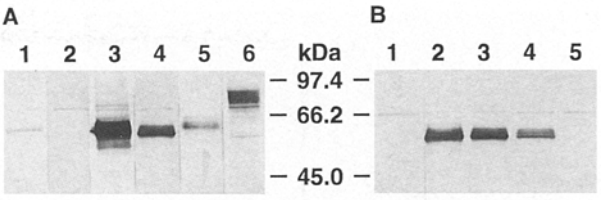


**Fig. 1A.** Western blot analysis of NaPi-2 protein expressed in Sf9 cells. After 5 days, infected cells were harvested and lysed with RIPA buffer (*see* Materials and Methods). Cell lysates (0.013 mg) were separated by SDS-PAGE and analyzed using anti(NaPi-2)antibodies raised against synthetic C- and N-terminal peptides [7]. Lane 3 and 5 anti N-terminal; Lane 4, anti C-terminal sera. Lane 5 contained whole cell lysate (0.013 mg) of Sf9 cells infected with the 6His-tagged NaPi-2 protein. Lanes 1 and 2 contained cell lysates (0.013 mg) of noninfected cells and were probed with anti N-terminal (Lane 1) and anti C-terminal (Lane 2) antibodies. Lane 6 contained 35 μg of purified rat kidney cortex brush-border membranes. (*B*) Time course of NaPi-2 expression in Sf9 cells: Cell lysates (0.013 mg) of infected cells were obtained after day 3,4,5 and 6 postinfection (Lanes 1 to 4); as a control Lane 5 contained cell lysate (0.013 mg) of noninfected cells. Western blots were performed with anti C-terminal antibodies.

Examination of NaPi-2 related immunofluorescence of infected Sf9 cells by confocal microscopy revealed intense staining in infected but not in noninfected Sf9 cells (Fig. 2*B* and *C*). Serial images of infected cells (Fig. 2*D*) showed that the NaPi-2 protein was located throughout the whole small cytoplasmic space surrounding the nucleus (Fig. 2*D;* middle row). No distinct plasmamembrane associated staining was observed.

### Functional Expression of Na/$P_i$-cotransport

To determine if the NaPi-2 protein when expressed in Sf9 cells is functionally active, i.e., exhibits Na-dependent transport of $P_i$, uptake of phosphate was determined in infected and noninfected cells. As illustrated in Fig. 3, Sf9 cells infected with the wild-type NaPi-2 protein exhibited a manyfold higher Na-dependent transport of $P_i$ compared to noninfected cells while Na-independent $P_i$-transport in infected cells was not different compared to noninfected cells. A stimulation of Na/$P_i$-cotransport was also observed in Sf9 cells infected with the 6His-tagged NaPi-2 protein (Fig. 3). In both cases, linear uptake rates of Na/$P_i$-cotransport were observed within the first 8 min of incubation (*not shown*).

Na-dependent $P_i$-transport in Sf9 cells infected with wild-type NaPi-2 protein was characterized in terms of the apparent $K_m$s of the interactions with phosphate and sodium ions and additionally by its dependence on the external pH-value (Fig. 4*A-C*). In infected cells, the apparent $K_m$ for $P_i$ was found to be in the order of 0.1 mM (*see* figure legend) whereas in noninfected cells, no saturation of Na/$P_i$-cotransport was observed up to 1 mM of external $P_i$. Interaction of expressed NaPi-2 transporters

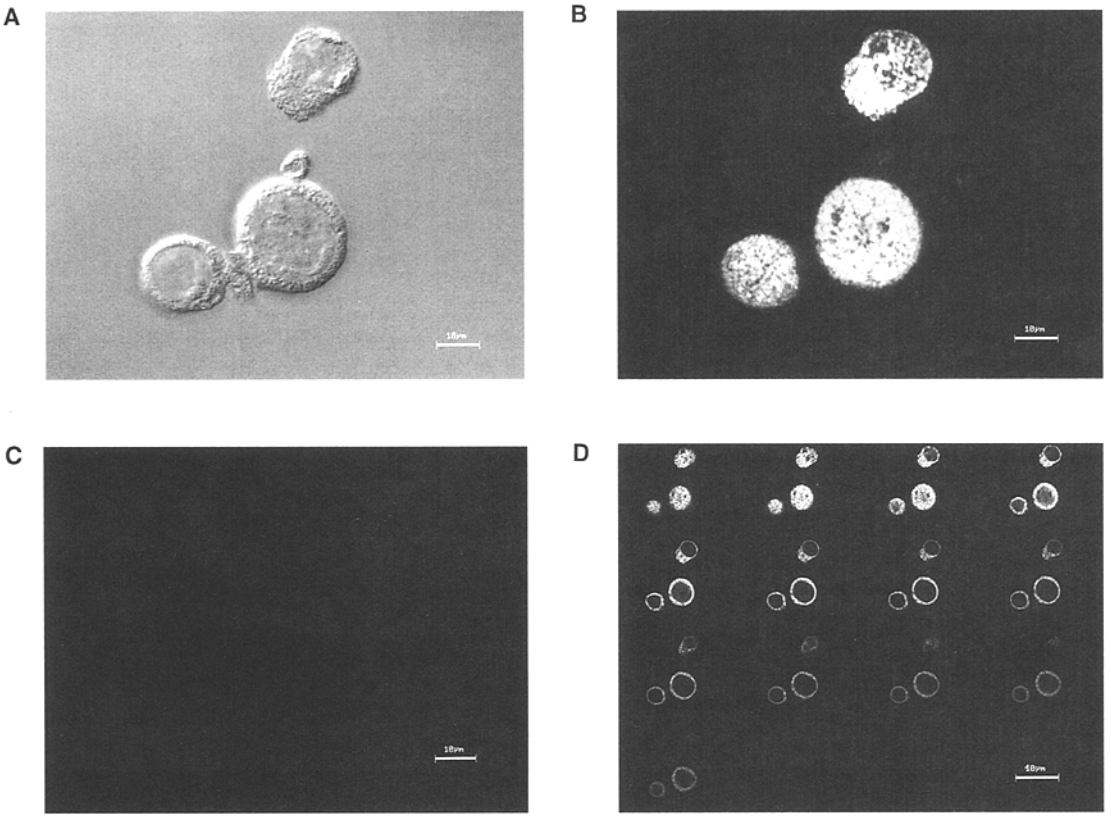


**Fig. 2.** Immunolocalization of wild-type NaPi-2 in Sf9 cells. Expressed NaPi-2 protein was examined after 4 days postinfection by indirect immunofluorescence using anti N-terminal antibodies. Micrographs were obtained by confocal microscopy. (*A*) Interference contrast microscopy of NaPi-2 infected Sf9 cells. (*B*) NaPi-2 mediated immunofluorescence; the focal plane was set above the nucleus. (*C*) Immunofluorescence of noninfected cells. (*D*) Serial images (steps of 1μ from top to bottom of the cells, start upper left) of NaPi-2 infected cells.

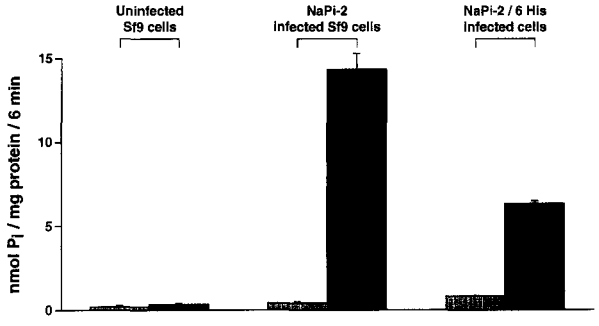


**Fig. 3.** Uptake of inorganic phosphate ($P_i$) of noninfected and infected Sf9 cells. Transport of $P_i$ was determined 4 days postinfection at room temperature for 6 min either in the presence of sodium (filled bars) or in the absence of sodium (shadowed bars). Data are given as the mean ± SD of four individual dishes obtained from one single infection.

with external sodium showed an apparent $K_m$ for sodium of 60 mM (*see* figure legend) and was highly sigmoidal with a calculated Hill coefficient of around 3, which would be in agreement with an electrogenic transport of $P_i$ as has been demonstrated recently in oocytes of *Xenopus laevis* injected with NaPi-2 cRNA [6]. The rate of Na/$P_i$-cotransport in infected Sf9 cells was stimulated by a more alkaline pH value of the external medium being in agreement with results obtained with oocytes of *X. laevis* injected with NaPi-2 cRNA [16].

## Discussion

We have documented that the recently cloned rat renal Na/$P_i$-cotransporter NaPi-2 [16], a member of renal type II Na/Pi-cotransporters [4], can be expressed in the Sf9 cell line by using the baculovirus system and that both the expressed NaPi-2 wild-type and 6His-tagged protein exhibit Na/$P_i$-cotransport. Expressed NaPi-2 mediated Na/$P_i$-cotransport in infected Sf9 cells was characterized with respect to the apparent constants of the interactions of the transporter with external $P_i$ and sodium and additionally by its dependence on the external pH-value. The results obtained by these transport studies suggest that the functionally active NaPi-2 protein as expressed in Sf9 cells is in all respects similar to Na/$P_i$-cotransport as observed in isolated proximal tubular brush-border membranes (1, 11; for review *see* ref. 17) and to the Na/$P_i$-

cotransport activity observed in oocytes of *X. laevis* injected with NaPi-2 cRNA [6, 16]. Furthermore, the Sf9 expression data suggest that the NaPi-2 related protein is indeed a transport system itself and not a cofactor required to activate silent activity in e.g., the oocytes and in Sf9 cells since it seems unlikely that in both systems such an activation of a "silent" activity would occur.

On immunoblots, the NaPi-2 protein as expressed in Sf9 cells was detected as a 65 kDa protein by both, anti(C-terminal) and an anti(N-terminal) anti(NaPi-2)antiserum indicating that the whole protein as predicted by the open reading frame [16] is made. In contrast, in proximal tubular brush-border membranes the mature NaPi-2 protein appears as a protein of 80 to 90 kDa (Fig. 1 and ref. 7). The most likely explanation for this observation is that the NaPi-2 protein as expressed in Sf9 cells is not or is poorly glycosylated, since the same mobility on SDS-PAGE has also been observed for the NaPi-2 protein after in vitro translation in the absence of microsomes [16] and after deglycosylation by endoglycosidase F of renal BBMV [10]. Although some partial glycosylation of the NaPi-2 protein can not be entirely excluded, it appears that the unglycosylated NaPi-2 protein still can fold properly, such that Na/$P_i$-cotransport is not impaired. In agreement, we recently found using site directed mutagenesis and subsequent injection of the mutated NaPi-2 cRNA into *X. laevis* oocytes, that *N*-glycosylation of the NaPi-2 protein is not necessary for a fully active Na/$P_i$-cotransport [10]. Similarly, a lack of glycosylation of other transport proteins expressed in Sf9 cells without affecting transport functions has been described by other laboratories [20, 23].

Exhibition of NaPi-2 related Na/$P_i$-cotransport in infected Sf9 cells suggests that the NaPi-2 protein is to some extent inserted into the plasma membrane of Sf9 cells. Interestingly, the rate of Na/$P_i$-cotransport (nmole $P_i$/mg total protein per min) in infected Sf9 cells was comparable to the rate of Na/$P_i$-cotransport observed in confluent OK-cells, an established renal epithelial cell line [3, 19]. Assuming the same translocation rate this would suggest that the amount of functional NaPi-2 transporters residing in the plasma membrane of Sf9 cells would be similar as in OK-cells. On the other hand however, by a quantitative comparison of the NaPi-2 abundance in isolated proximal tubular brush-border

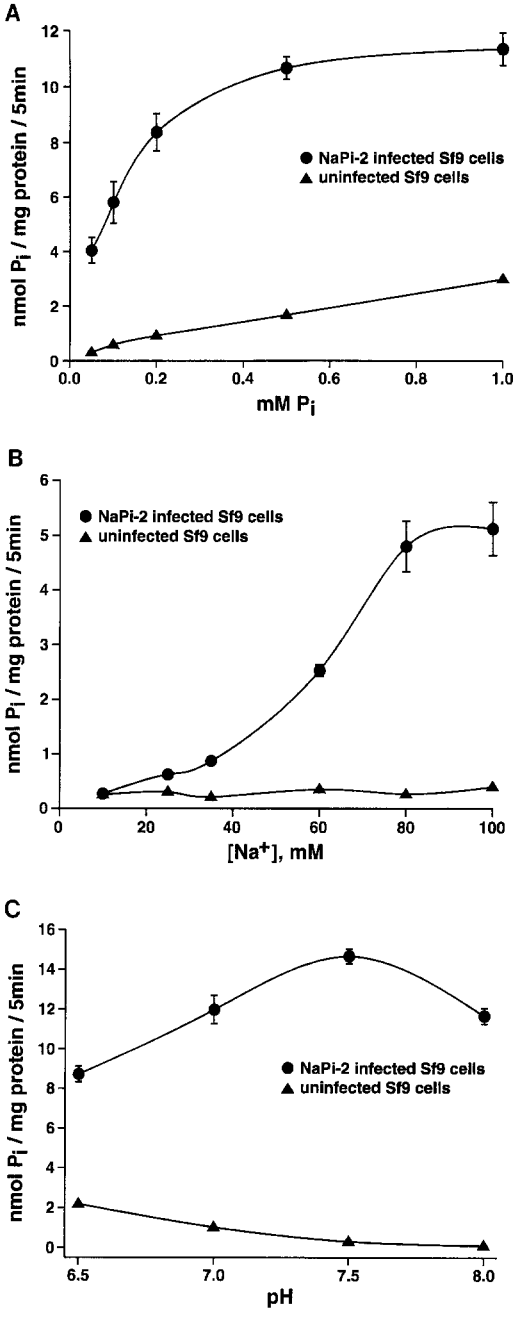


**Fig. 4.** Characterization of Na/$P_i$-cotransport mediated by wild-type NaPi-2 expressed in Sf9 cells. Transport experiments were performed at room temperature. The data represent the mean ± SD of four dishes obtained by one infection. (*A*) Four days after infection, $P_i$-uptake was measured within linear uptake conditions as a function of $P_i$ concentration. The curve was fitted according to the Lineweaver-Burk equation ($K_m$ = 0.114 ± 0.009 mM; $V_{max}$ = 12.75 ± 0.3 nmol $P_i$ · (mg protein · 5 min)$^{-1}$. (*B*) Four days after infection transport was measured in the presence of various concentrations of $Na^+$. The Hill coefficient was obtained by curve fitting on the basis of the Hill equation ($K_m$ = 63 ± 8 mM; $V_{max}$ = 5.6 ± 4.3 nmol $P_i$ · (mg protein · 5 min)$^{-1}$ and $n$ = 3 ± 0.9). (*C*) $P_i$-uptake at various external pH values.

[1] Quantitative analysis of the Western blot signals as illustrated in Fig. 1 revealed that the abundance of the NaPi-2 protein in Sf9 cells (in 0.013 mg of total cellular protein) is twofold higher compared to the purified BBMV's (37 µg protein). Assuming that brush-border membranes represent about 5% of the total protein of a proximal tubular cell 37 µg of BBMV protein would correspond to about 0.74 mg of total proximal cell protein. Thus expression of the NaPi-2 protein per mg of total cellular protein would be approximately 100-fold higher in Sf9 cells compared to proximal tubular cells.

membranes and cell lysates of infected Sf9 cells it was estimated that the expression of the wild-type NaPi-2 protein in infected Sf9 cells may be approximately 100-fold higher than in proximal tubular cells.[1] Still the expressed NaPi-2 protein did not appear as a prominent protein on Coomassie blue stained gels (*data not shown*), as described for expression of the Na/H-exchanger NHE-1 [9] in Sf9 cells. As observed by immunofluorescence, it was evident that most of the NaPi-2 protein was localized within the small cytoplasmic space of Sf9 cells. Therefore it is concluded, that although total expression of NaPi-2 protein in Sf9 cells is roughly 100-fold higher than in proximal cells, the extent of insertion into the plasma membrane of Sf9 cells of NaPi-2 cotransporters does not exceed levels found, for example, in OK-cells.

The possibility of expressing the renal $Na/P_i$-cotransporter NaPi-2 as a 6His-tagged protein, which is functionally active, may allow a purification of this protein in quantity and thereby make it available for further studies on structure/function relationships.

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*Note added in proof:* To introduce 6 histidine residues at the N-terminal end of the NaPi-2 protein the vector pAcSG-His NT-B as obtained from Pharmingen (San Diego, CA) was used. In subsequent experiments we could however not verify that the expressed NaPi-2 was indeed 6His tagged as predicted.

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